FINAL PROTOCOL

Testing Facility Study No. WIL-459501

An Oral (Drinking Water) Study of the Effects of Trichloroethylene (TCE) on Fetal Heart Development in Sprague Dawley Rats

SPONSOR:
Halogenated Solvents Industry Alliance, Inc
3033 Wilson Boulevard, Suite 700
Arlington, VA 22201
USA

TESTING FACILITY:
Charles River Laboratories Ashland, LLC
1407 George Road
Ashland, OH 44805
United States

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1. OBJECTIVE

The objective of this study is to determine the potential of the test substance to induce cardiac defects in the offspring after maternal exposure from the day after copulation to 1 day prior to expected parturition, to characterize maternal toxicity at the exposure levels tested and to determine a NOAEL (no-observed-adverse-effect level) for maternal and cardiac developmental toxicity.

Additionally, assessments of exposure levels of TCE, and its primary metabolite, trichloroacetic acid (TCA), in maternal and fetal plasma will be performed.

2. REGULATORY COMPLIANCE

This study will be conducted in compliance with the United States Environmental Protection Agency (EPA) TSCA (40 CFR Part 792) Good Laboratory Practice Standards and the Organisation of Economic Cooperation and Development Guidelines (OECD) [C(97) 186/Final] Principles of Good Laboratory Practice.

The study will be performed according to the protocol and protocol amendments, if any, as approved by the Sponsor. Portions of the study conducted at Charles River will be performed according to Charles River Standard Operating Procedures.

3. REGULATORY GUIDELINES

This study will be conducted in general accordance with the United States Environmental Protection Agency (EPA) Health Effects Test Guidelines OPPTS 870.3700, Prenatal Developmental Toxicity Study, August, 1998 and the Organisation of Economic Co-operation and Development Guidelines (OECD) for the Testing of Chemicals Guideline 414, Prenatal Developmental Toxicity Study, January 2001.

4. PERSONNEL INVOLVED IN THE STUDY

4.1. Sponsor Representative

John U. Bell, PhD, DABT
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4.2. Sponsor Study Monitor

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4.3. Charles River Study Director

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Director, Developmental and Reproductive Toxicology
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4.4. Charles River Alternate Contact

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Staff Toxicologist, Developmental and Reproductive Toxicology/Neurosciences
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5. PROPOSED STUDY SCHEDULE

Experimental Starting (Animal Receipt) Date:	11 October 2016
Experimental Start (First Day of Dosing) Date:	19 October 2016
Experimental Completion/Termination Date:	11 November 2016
Audited Draft Report Mail Date:	25 January 2017

6. TEST SUBSTANCE, POSITIVE CONTROL SUBSTANCE AND VEHICLE DATA

6.1.1. Test Substance

6.1.2. Identification

Trichloroethylene (TCE) (CAS No. 79-01-6) ≥99% and scavenger-free

Purchased from Spectrum Chemical Manufacturing Corp. (T1115 reagent grade, or equivalent).

6.1.3. Characterization

Lot numbers, purity, stability, and storage conditions will be provided by the Supplier/Manufacturer, documented in the study records and included in the Final Report.

6.1.4. Storage Conditions

In a room with controls set to maintain 18°C to 24°C, protected from light.

6.1.5. Physical Description

To be documented by Charles River.

6.1.6. Reserve Samples

Reserve samples of the test substance will be taken in accordance with Charles River Standard Operating Procedures and stored in the Charles River Archives indefinitely, unless otherwise specified.

6.1.7. Personnel Safety Data

A Material Safety Data Sheet (MSDS) is to be provided by the Supplier/Manufacturer. Standard safety precautions will apply.

6.1.8. Test Article Disposition

With the exception of the reserve sample for each batch of test article (if applicable), all neat test article remaining at study completion will be discarded appropriately.

6.2. Positive Control Substance

6.2.1. Identification

all-*trans* Retinoic Acid ≥98% by HPLC (CAS No. 302-79-4)

Purchased from Sigma-Aldrich, Inc. (R2625, or equivalent)

6.2.2. Characterization

Lot numbers, purity, stability, and storage conditions will be provided by the Supplier/Manufacturer, documented in the study records and included in the Final Report.

6.2.3. Storage Conditions

In a freezer, set to maintain -10ºC to -30°C, protected from light.

6.2.4. Physical Description

To be documented by Charles River

6.2.5. Reserve Samples

Reserve samples of the positive control substance will be taken in accordance with Charles River Standard Operating Procedures and stored in the Charles River Archives indefinitely, unless otherwise specified.

6.2.6. Personal Safety Data

A Material Safety Data Sheet (MSDS) is to be provided by the Supplier/Manufacturer. Standard safety precautions will apply.

6.3. Vehicle (for Drinking Water Formulations)

6.3.1. Identification

Reverse osmosis-purified water

6.3.2. Characterization

Water used on-site is subject to routine monitoring as indicated in SOP A-067. Standard safety precautions will apply.

6.4. Vehicle (for Positive Control Formulations)

6.4.1. Identification

Soybean oil (CAS No. 8001-22-7)

Purchased from Sigma-Aldrich, Inc. (S7381 dietary grade, or equivalant)

6.4.2. Characterization

Lot numbers, purity, stability, and storage conditions will be provided by the Supplier/Manufacturer, documented in the study records and included in the Final Report.

7. PREPARATION AND ANALYSIS OF TEST AND POSITIVE CONTROL SUBSTANCE FORMULATIONS

7.1. Test Substance Formulations

7.1.1. Method and Frequency of Preparation

Based on the physical characteristics of the test substance, appropriate methods will be used to ensure the best possible formulations of the test substance in the vehicle. **Test substance formulations will be prepared under amber light and stored and transported in large amber bottles for light protection.** Test substance formulations will be prepared daily and stored at room temperature (18°C to 24°C) for a period not to exceed established stability.

Any procedures not covered by SOPs required for formulation will be added to the protocol by protocol amendment and presented in the final report of this study.

The Study Director or designee will visually inspect the test substance formulations prior to initiation of dosing. This visual inspection will be performed to ensure that the formulations are visibly homogeneous and acceptable for dosing.

7.1.2. Homogeneity and Stability of Test Substance in Drinking Water Formulations

Test substance formulations will be analyzed using a method being developed and validated on a concurrent study.[1] Homogeneity/solubility and stability of the test substance in the vehicle following room temperature (18°C to 24°C) for up to 24 hours at the range of concentrations being used on the current study will also be established on the same study. Therefore, stability and homogeneity of test article formulations will not be assessed on this study.

7.1.3. Concentration of Test Substance in Drinking Water Formulations

Concentration of test substance in dosing formulations, including the vehicle control, will be assessed on the first and last batch of formulations used to dose all groups. For concentration assessment, four 1.0 mL samples will be collected from the middle of the control and each test substance formulation. Two samples will be analyzed to assess the concentration of the test substance in the formulations; the remaining samples will be stored at room temperature (18°C to 24°C) as backup samples, which may be analyzed at the request of the Study Director.

The acceptable result for concentration assessment of a solution is a mean concentration within 100% ± 10% (90-110%) of the target concentration.

Any backup samples kept at Charles River will be discarded following acceptance of the analytical results by the Study Director.

The final analytical report will be incorporated as an appendix to the Charles River final report.

7.2. Positive Control Substance

7.2.1. Method and Frequency of Preparation

Based on the physical characteristics of the positive control substance, appropriate methods will be used to ensure the best possible formulations in the vehicle, soybean oil, which may be warmed to ensure solubilization, if necessary. **Positive control substance formulations will be prepared under amber light and stored and transported in small amber aliquot bottles for light protection.** Positive control substance formulations will normally be prepared approximately weekly, divided into aliquots for daily dispensation, purged with nitrogen and stored in a freezer, set to maintain -10ºC to -30°C. The positive control formulations will be thawed for each day of administration, and dispensed after remixing for a minimum of 30 minutes using a magnetic stirrer. Positive control formulations will be stirred continuously during dosing.

Any procedures not covered by SOPs required for formulation will be added to the protocol by protocol amendment and presented in the final report of this study.

7.2.2. Concentration of Positive Control Substance in Soybean Oil Formulations

Positive control formulations in the vehicle, soybean oil, will not be assessed for solubility, concentration, homogeneity, or stability. All-*trans* retinoic acid (RA) is a commercially available drug substance that will be prepared according to package specifications. It is a well characterized developmental toxicant that has been previously demonstrated to result in heart malformations in this strain of rat.[2]

For future possible assessment of concentration of the positive control substance in dosing formulations, duplicate 1.0 mL samples will be collected from the middle strata on the first and last day of use for each batch of dosing formulations. Samples will be purged with nitrogen and stored in a freezer, set to maintain -10ºC to -30°C for future possible analytical assessments.

8 TEST SYSTEM

8.1 Species

Rat

8.2 Strain

Sprague Dawley Crl:CD(SD)

8.3 Source

Charles River Laboratories, Inc.
(Facility to be documented in the study records)

8.4 Number of Study

One hundred and seventy (170) females (maximum of 212 purchased). A sufficient number of sexually mature untreated resident males of the same strain and source will be used to induce pregnancies. Animals not assigned to the study will be transferred to the animal colony or will be euthanized by carbon dioxide inhalation and the carcasses discarded.

The number of animals is based on the US EPA Health Effects Test Guidelines OPPTS 870.3700, Prenatal Development Toxicity Study, August 1998 and the OECD Guidelines for the Testing of Chemicals: Guideline 414, Prenatal Developmental Toxicity Study, January 2001, which recommend evaluation of approximately 20 females with implantation sites at necropsy. Given the possibility of nongravid animals, unexpected deaths, or treatment-related moribundity and/or mortality, this is an appropriate number of animals to obtain a sample size of 20 at termination.

8.5 Body Weight Range

A minimum of 220 g at initiation of breeding.

8.6 Approximate Age

Eighty to 120 days at the initiation of breeding.

8.7 Identification System

A permanent animal number will be assigned to each individual animal upon receipt. This permanent animal number will be programmed into a microchip (BMDS system). The microchip will be implanted subcutaneously in the dorsoscapular region for each individual animal during the acclimation period. The microchip will be the primary means to uniquely identify animals assigned to study. Individual cage cards will be affixed to each cage and will display at least the animal number, cage number, group number, study number, dosage level, and sex of the animal.

Replacement microchips may be implanted as necessary throughout the course of the study. If the condition of the implantation site prevents the use of a microchip, a Monel® metal ear tag may be used as the unique identifier.

8.8 Justification for Selection

This species and strain of rat has been recognized as appropriate for developmental toxicity studies. Charles River Ashland has historical data on the background incidence of fetal malformations and developmental variations in the Crl:CD(SD) rat. This animal model has been proven to be susceptible to the effects of developmental toxicants.

9 SPECIFIC ANIMAL MAINTENANCE SCHEDULE

9.1 Animal Receipt and Acclimation

Each rat will be inspected by a qualified technician upon receipt. Rats judged to be in good health and suitable as test animals will be immediately placed in acclimation for a minimum of 7 days. All rats will be initially weighed, permanently identified with a microchip and receive a clinical observation. During the acclimation period, each rat will be observed twice daily for changes in general appearance and behavior. Body weights will be recorded prior to the initiation of breeding. Prior to the start of the in-life phase, those rats judged to be suitable test subjects will be identified.

During social housing, some observations (*e.g.*, fecal observations) may not be attributable to an individual animal. In these instances, observations will be recorded in a separate computer file for the social group.

9.2 Animal Housing

All females will be housed in groups of 2-3 following receipt and throughout acclimation in clean, solid-bottom cages with bedding material (Bed O'Cobs®) or other suitable material in an environmentally controlled room. Following positive signs of mating, each female will be individually housed in clean, solid-bottom cages with bedding material (Bed O'Cobs®) or other suitable material in an environmentally controlled room. The cages will be subjected to routine cleaning at a frequency consistent with maintaining good animal health and Charles River Standard Operating Procedures. The facilities at Charles River are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International).

Individual housing of presumed pregnant females is required to adequately monitor the health of these females by allowing collection of individual food and water consumption and appropriate identification of cage observations in the event of abortion or early delivery.

9.3 Environmental Conditions

Controls will be set to maintain temperature at 73 ± 5°F (23°C ± 3°C) and relative humidity at 50 ± 20%. Temperature and relative humidity will be monitored continuously. Data for these 2 parameters will be scheduled for automatic collection on an hourly basis. Fluorescent lighting controlled by light timers will provide illumination for a 12-hour light/dark photoperiod. The ventilation rate will be set at a minimum of 10 room air changes per hour, 100% fresh air.

9.4 Drinking Water

Cage banks will not be connected to the automated watering system. Reverse osmosis-purified water (with test substance added during the treatment period for animals assigned to Groups 3-6) will be available *ad libitum* via amber glass water bottles with metal sipper tubes. Bottles will be checked daily for spillage and supplemented as necessary and the occurrence of spillage will be documented. During the treatment period, bottles will be changed daily. The municipal water supplying the laboratory is analyzed according to Charles River Ashland SOPs on a routine basis to ensure that contaminants are not present in concentrations that would be expected to affect the outcome of the study.

9.5 Basal Diet

PMI Nutrition International, LLC Certified Rodent LabDiet® 5002 will be offered *ad libitum* during the study. Periodic analyses of the certified feed are performed by the manufacturer to ensure that heavy metals and pesticides are not present at concentrations that would be expected to affect the outcome of the study. Results of the analyses are provided to Charles River by the manufacturer. Feeders will be changed and sanitized once per week.

9.6 Environmental Enrichment

Enrichment devices will be provided to each animal for environmental enrichment and to aid in maintaining the animals' oral health, beginning during acclimation, and continuing throughout the course of the study.

10 EXPERIMENTAL DESIGN

10.1 Breeding Procedure

At the conclusion of the acclimation period, female rats judged to be suitable test subjects and meeting acceptable body weight requirements will be cohabitated with untreated resident male rats (1:1) of the same strain and source in solid-bottom cages for mating. Detection of mating will be confirmed by the appearance of a vaginal copulatory plug or by evidence of sperm in a vaginal lavage. Vaginal lavages will be performed daily during the mating period until evidence of mating is observed. After confirmation of mating, the female will be returned to an individual solid bottom cage (assigned to a group), and the day will be designated as day 0 of gestation.

10.2 Animal Selection and Randomization

Mated females will be assigned to groups using a WIL Toxicology Data Management System (WTDMS™) computer program which assigns animals based on stratification of gestation day 0 body weights into a block design to 1 control group and 4 test substance groups of 25 rats each for the prenatal developmental (Main) phase. Rats will also be assigned to 1 positive control group of 25 rats. In addition, animals will also be assigned to 1 control group and 4 test substance groups of 4 rats each for the exposure assessment (Exp) phase.

Following the initiation of dosing, it may be necessary to add individual animal(s) (due to animals being found dead, euthanized *in extremis*, exhibiting abnormal clinical signs, reduced food consumption, body weigh losses, or dosing errors). Individual animals that are added to the study will be selected from the remaining unassigned animals, and assigned arbitrarily (not computer randomized) to the study based on comparable body weights (if possible) with respect to the animal(s) previously assigned to the study. The reason(s) for adding the animal(s) will be appropriately documented in the study records.

10.3 Organization of Test Groups, Dosage Levels, and Treatment Regimen

10.3.1 Rationale for Dose Selection

The dosage levels were selected based on previous published reports assessing fetal heart development in Sprague Dawley rats[2, 3, 4] and were provided by the Sponsor Representative after consultation with the Charles River Study Director.

The positive control substance, RA, is a well characterized developmental toxicant that has been previously demonstrated to result in heart malformations in this strain of rat. The dosage level of was selected also based on previously published reports.[2]

10.3.2 Organization of Test Groups

The following table presents the study group arrangement.

Study Design

Group Number	Test Substance	Dosage Level (mg/kg/day)	Dose Concentration	Dose Volume (mL/kg)	Route of Administration	Number of Females	
						Main	Exp
1	Vehicle control	0	0 ppm	NA	Drinking Water	25	4
2	RA	15	3 mg/mL	5	Gavage	25	0
3	TCE	a	0.25 ppm	NA	Drinking Water	25	4
4	TCE	a	1.5 ppm	NA	Drinking Water	25	4
5	TCE	a	500 ppm	NA	Drinking Water	25	4
6	TCE	a	1000 ppm	NA	Drinking Water	25	4

[a] dosage levels for the drinking water groups will be calculated upon completion of the study based on mean water consumption and mean body weights during the course of the study.

10.3.3 Route and Rationale of Test and Positive Control Substance Administration

The route of administration of the test substance will be oral (drinking water) as this is a potential route of exposure for humans. Historically, this route has been used extensively for studies of this nature. The positive control substance, RA, will be administered via oral (gavage) as that route of exposure has been demonstrated to elicit a positive response.[2]

10.3.4 Treatment Regimen - Test and Positive Control Substances

Vehicle control or test substance drinking water formulations will be offered *ad libitum* from gestation day 1 through euthanasia. Water formulations will be supplied fresh on a daily basis.

The positive control substance will be administered as a single daily dose from gestation day 6 through 15, inclusively (Group 2 only). This is the standard dosing regimen for a prenatal developmental toxicity study and is expected to elicit a positive response.[2] All rats will be dosed at approximately the same time each day.

10.3.5 Method of Test and Positive Control Substance Administration

Control and treated drinking water formulations will be offered *ad libitum* in amber glass water bottles with metal sipper tubes. Water bottles will be changed and sanitized daily, and water formulations will be supplied fresh on a daily basis.

The positive control substance will be administered orally by gavage (Group 2 only) using appropriately sized disposable plastic feeding tubes (Instech Solomon, Plymouth Meeting, PA). The dose volume will be 5 mL/kg. Formulations will be stirred continuously at room temperature for the duration of the dosing procedure.

10.3.6 Adjustment of Doses/Dose Volumes

The test substance will be administered as a constant concentration (ppm) in water.

For the positive control substance treated group (Group 2), individual dosages will be calculated on the most recent body weight to provide the proper mg/kg/day dosage.

11 PARAMETERS TO BE EVALUATED

11.1 Viability Observations

Each rat will be observed twice daily for moribundity and mortality, once in the morning and once in the afternoon from gestation day 0 until euthanasia.

11.2 Clinical Observations During Gestation

Clinical observations will be recorded daily. Mortality and all signs of overt toxicity will be recorded on the day observed. The observations shall include, but are not limited to, evaluation for changes in appearance of skin and fur, eyes, mucous membranes, respiratory and circulatory system, autonomic and central nervous systems, somatomotor activity, and behavior. All animals will also be observed on the day of necropsy and any findings will be recorded.

For the positive control substance treated group (Group 2 only), each animal will be observed approximately 1 hour following each dose administration for findings that are potentially related to treatment or that might change before the next scheduled observation. Additional post-dosing observations may be necessary and will be documented in the study records.

11.3 Body Weights

Individual body weights will be recorded on gestation days 0-21 (daily) for animals assigned to the main and exposure assessment phases.

11.4 Water Consumption

Individual water consumption (by weight) will be recorded on gestation days 0-21 (daily) for animals assigned to the main and exposure assessment phases.

11.5 Food Consumption

Individual food consumption will be recorded on gestation days 0-21 (daily) for animals assigned to the main phase. Food intake will be reported as g/animal/day and g/kg/day for each corresponding body weight interval of gestation.

Food consumption will not be recorded for animals assigned to the exposure assessment phase.

11.6 Deaths and Animals Euthanized *in Extremis*

Females not surviving until the scheduled euthanasia will be necropsied (as soon as possible upon discovery) and cause of death recorded, if possible. Rats not expected to survive to the next observation period (moribund) will be euthanized by carbon dioxide inhalation. The cranial, thoracic, abdominal, and pelvic cavities will be opened and the organs examined. The number and location of implantation sites and viable fetuses will be recorded. Corpora lutea will also be counted and recorded. Uteri which appear nongravid by macroscopic examination will be opened and placed in 10% ammonium sulfide solution for detection of early implantation loss.[5] Gross lesions will be preserved in 10% neutral-buffered formalin for possible future histopathologic examination. Carcasses from adult animals will be discarded. Viable fetuses will be euthanized by a subcutaneous injection of sodium pentobarbital in the scapular region. Recognizable fetuses will be examined externally and preserved in 10% neutral-buffered formalin.

Animals dying or euthanized *in extremis* (by carbon dioxide inhalation) that are assigned to the exposure assessment phase will have pregnancy status determined (by ammonium sulfide, if necessary). Viable fetuses will be euthanized by a subcutaneous injection of sodium pentobarbital in the scapular region. Carcasses of the dams and fetuses will be discarded.

11.7 Premature Deliveries

Females that deliver prematurely will be euthanized by carbon dioxide inhalation that day. The thoracic, abdominal, and pelvic cavities will be opened and the organs examined. The number and location of former implantation sites and viable fetuses will be recorded. Corpora lutea will also be counted and recorded. Gross lesions will be preserved in 10% neutral-buffered formalin for possible future histopathologic examinations. Carcasses from adult animals will be discarded. Viable fetuses or pups will be euthanized by a subcutaneous (scapular region) or intraperitoneal injection of sodium pentobarbital (as appropriate). Recognizable fetuses or pups will be examined externally and preserved in 10% neural buffered formalin. Recognizable fetuses or pups aborted on GD 21 will be examined according to the fetal examination section (Section 14.2), if possible.

Females that deliver prematurely that are assigned to the exposure assessment phase will be euthanized by carbon dioxide inhalation that day and identified as gravid. Viable pups will be euthanized by an intraperitoneal injection of sodium pentobarbital. Carcasses of the dams and pups will be discarded.

12 SAMPLE COLLECTION AND SCHEDULED NECROPSY FOR THE EXPOSURE ASSESSMENT PHASE

12.1 Intervals

Dams: Gestation days 8, 12 and 21

Fetuses: Gestation Day 21

12.2 Blood Collection Time Points

Dams (Gestation Day 8 and 12): A single blood samples will be collected from each dam between 0830 and 1130 hours.

Dams and Fetuses (Gestation Day 21): A single blood sample will be collected from each dam just prior to euthanasia. Immediately following blood collection, each dam will be euthanized by carbon dioxide inhalation and uteri which appear gravid by macroscopic examination will be removed for fetal blood collection.

12.3 Number of animals

Dams: Four (4) females/group assigned to the exposure assessment phase.

Fetuses: Up to 4 litters (pooled) per group from dams assigned to the exposure assessment phase.

12.4 Method/Route of Collection

Dams: via the jugular vein using the hand-held restraint method.

Fetuses: via the umbilical vein of each fetus.

12.5 Target Blood Volume

Dams: 0.5 mL/animal/time point; collection in pre-chilled, uniquely labeled amber tubes. Samples will be protected from light to the extent possible.

Fetuses: As much blood as possible; blood will be pooled by litter regardless of sex, in pre-chilled, uniquely labeled amber tubes. Samples will be protected from light to the extent possible.

12.6 Anticoagulant

Lithium Heparin (amber tubes)

12.7 Sample Handling and Plasma Preparation

Samples will be kept on wet ice, protected from light, following blood collection and through centrifugation, plasma collection, and storage. All samples will be centrifuged (approximately 3000 rpm [approximately 2056xg] for approximately 10 min) at approximately 4°C.

12.8 Aliquots

The maximum amount of plasma will be recovered and plasma will be transferred into new, uniquely-labeled amber polypropylene tubes.

12.9 Label Information

Samples, and/or accompanying paperwork, will include study number, dose group, animal number, gestation day interval, number of pups (in pooled samples), sample type, and date and time of blood collection.

12.10 Storage

Plasma samples will be stored in a freezer set to maintain -65°C to -85°C until transferred to the Charles River Bioanalytical Chemistry Department for analysis using a method being developed and validated on a concurrent study.[6] The time and date that the samples are placed in the freezer will be recorded.

12.11 Disposition of Animals/Laparotomy

All exposure assessment phase rats will be euthanized by carbon dioxide inhalation following the last blood collection (GD 21). Uteri which appear gravid by macroscopic examination will be removed immediately for fetal blood collection and the dams will be identified as gravid. Uteri which appear nongravid by macroscopic examination will be opened and placed in 10% ammonium sulfide solution for detection of early implantation loss.[5] Following blood collection, fetuses will be euthanized by decapitation. Carcasses of the dams and fetuses will be discarded without further examination.

12.12 Sample Transfer for Bioanalysis

Plasma samples, an inventory list and documentation of actual blood collection times for each animal, will be transferred to the Charles River Bioanalytical Chemistry Department for analysis of TCE and TCA concentrations.

Any remaining samples kept at Charles River will be discarded following acceptance of the bioanalytical results by the Study Director.

The bioanalytical report will be included as an appendix to the Charles River final report.

13 EXPOSURE ASSESSMENT

An exposure assessment report for maternal and fetal mean plasma concentration values of TCE and TCA at each dosage level and interval will be incorporated as an appendix to the Charles River final report. A summary of the exposure data will be incorporated into the text of the final report as appropriate.

14 SCHEDULED NECROPSY - GESTATION DAY 21

14.1 Laparohysterectomy and Macroscopic Examination

Laparohysterectomy and macroscopic examinations will be performed blind to treatment group. All surviving rats will be euthanized by carbon dioxide inhalation on gestation day 21. The thoracic, abdominal, and pelvic cavities will be opened and the organs examined. The uterus of each dam will be excised and its adnexa trimmed. Corpora lutea will be counted and recorded. Gravid uterine weights will be obtained and recorded. The uterus of each dam will be opened and the number of viable and nonviable fetuses, early and late resorptions, and total number of implantation sites will be recorded, and the placentae will be examined. The individual uterine distribution will be documented using the following procedure: all implantation sites, including early and late resorptions, will be numbered in consecutive fashion beginning with the left distal uterine horn, noting the position of the cervix and continuing from the proximal to the distal right uterine horn. Uteri which appear nongravid by macroscopic examination will be opened and placed in a 10% ammonium sulfide solution for detection of early implantation loss.[5] Maternal tissues will be preserved for future histopathologic examination in 10% neutral-buffered formalin only as deemed necessary by the gross findings. Representative sections of corresponding organs from a sufficient number of controls will be retained for comparison, if possible. The carcasses will be discarded.

14.2 Fetal Examination

Fetal examinations will be conducted without knowledge of treatment group. All fetuses will receive an external examination. Internal (visceral) examination will be limited to an examination of the heart and great and major blood vessels only. Representative photographs of all cardiac and great and major blood vessel malformations, as appropriate, will be included in the study records. In addition, representative photographs of a normal littermate, will also be included in the study records, as needed and as appropriate, for comparison, where possible. **Representative photographs of all malformations with comparison photographs of normal fetuses will be included in the final report.** Prenatal data (viable and nonviable fetuses, early and late resorptions, pre- and post-implantation loss, and the fetal sex distribution) will be presented on a group mean basis and additionally as proportional data (% per litter).

14.2.1 External

Each viable fetus will be examined in detail, sexed, weighed, and euthanized by a subcutaneous injection of sodium pentobarbital in the scapular region. Nonviable fetuses (the degree of autolysis is minimal or absent) will be examined, crown-rump length measured, weighed, sexed and tagged individually. The crown-rump length of late resorptions (advanced degree of autolysis) will be measured, the degree of autolysis recorded, a gross external examination performed (if possible), and the tissue will be discarded.

14.2.2 Visceral (Internal)

Fetuses will be examined for visceral cardiac anomalies by dissection in the fresh (non-fixed) state. The thoracic cavity will be opened and dissected using a technique described by Stuckhardt and Poppe[7] with the exception that internal examination will be limited to a thorough examination of the heart and great and major blood vessels only. The abdomen will be opened with the sole purpose of internal confirmation of the sex of all fetuses. All carcasses will be discarded following completion of internal examination.

15 MAJOR COMPUTER SYSTEMS - DATA ACQUISITION, ANALYSIS, AND REPORTING

The major computer systems used on this study include, but are not limited to, the following systems.

All computerized systems used for data collection during the conduct of this study have been validated (with the exception of Microsoft Office and GraphPad Prism® 2008); when a particular system has not satisfied all requirements, appropriate administration and procedural controls were implemented to assure the quality and integrity of the data.

The actual version number will be specified in the report.

Critical Computerized Systems

Program/System	Description
Archive Management System (AMS)	In-house developed application for storage, maintenance, and retrieval of information for archived materials (*e.g.*, lab books, study data, wet tissues, slides, *etc.*).
Bio Medic Data Systems (BMDS) Implantable Programmable Transponders™ (IPT-300)	Animal identification
InSight® Publisher	Electronic publishing system (output is Adobe Acrobat, PDF).
Master Schedule	Maintains the master schedule for the company.
MD5 Checksum Tool	Used to generate and verify MD5 checksums during the final report generation process to create a significant, permanent link between the electronic study report and the signature page.
Metasys DDC Electronic Environmental Control System	Controls and monitors animal room environmental conditions.
Microsoft® Office 2010 or higher; GraphPad Prism® 2008	Used in conjunction with the publishing software to generate study reports.
Provantis Dispense™	Comprehensive system (Instem LSS Limited) to manage test materials, including receipt, formulation instructions, and accountability.
WIL Formulations Dispense System (WFDS)	In-house developed system for use in conjunction with Provantis Dispense™ to ensure proper storage and use of formulations.
WIL Metasys	In-house developed system used to record and report animal room environmental conditions.

Program/System	Description
WIL Toxicology Data Management System™ (WTDMS™)	In-house developed system used for collection and reporting of in-life and *postmortem* data.

Note: Version numbers of WTDMS™ programs used for the study are presented on the report data tables (reporting programs); version numbers and release dates are otherwise maintained in the study records and/or facility records.

16 STATISTICAL METHODS

All analyses will be two-tailed for significance levels of 5% and 1%. All statistical tests will be performed using a computer with appropriate programming as referenced below. Data from nongravid females will be excluded from calculation of means and from comparative statistics. The litter, rather than the fetus, will be considered as the experimental unit.

Comparative statistics will not be performed on in-life or necropsy data from exposure assessment phase animals.

Data for the positive control substance group will be compared to the control group using a two-sample t-test[8] to determine intergroup differences.

16.1 Maternal In-Life Data

Continuous data variables (maternal body weights [absolute and net], body weight gains [absolute and net], food, and water consumption of each interval) will be subjected to a parametric one-way analysis of variance (ANOVA).[9] If the results of the ANOVA are significant ($p<0.05$), Dunnett's test[10] will be applied to the data to compare the test substance treated groups to the control group.

16.2 Laparohysterectomy Data

The group mean numbers of corpora lutea, implantation sites, viable fetuses, maternal gravid uterine weights and mean fetal weight (separately by sex, and combined) will be subjected to a parametric one-way analysis of variance (ANOVA) and Dunnett's test as described above.[9,10] The mean litter proportions of prenatal data (% per litter of viable and nonviable fetuses, early and late resorptions, total resorptions, pre- and post-implantation loss, and the fetal sex distribution) will be subjected to the Kruskal-Wallis nonparametric ANOVA test[11] to determine intergroup difference. If the results of the ANOVA are significant ($p<0.05$), Dunn's test[12] will be applied to the data to compare the test substance treated groups to the control group.

16.3 Fetal Morphology Data

The mean litter proportion (% per litter) of total fetal cardiac malformations and developmental variations and of each particular visceral cardiac malformation or variation will be tabulated. The mean litter proportions of fetal cardiac malformations and developmental variations will be subjected to the Kruskal-Wallis nonparametric ANOVA test followed by Dunn's test (if

appropriate), to compare the test substance treated groups to the control group, as described above.[11,12]

17 QUALITY ASSURANCE

The study will be audited by the Charles River Quality Assurance (QA) Department in accordance with SOP QA-001 while in progress to assure compliance with Good Laboratory Practice Regulations, adherence to the protocol and amendments, if any, and to Charles River Standard Operating Procedures. The raw data and draft report will be audited by the Charles River QA to assure that the final report accurately describes the conduct and the findings of the study.

This study will be included on the Charles River master list of regulated studies.

Test site Quality Assurance responsibilities are described in the applicable appendices.

18 WORK PRODUCT

All original raw data records, as defined by Charles River SOPs and the applicable GLPs, will be stored in the Charles River Archives as described below. Storage and retention of records and materials by test sites for the delegated phases are described in the applicable appendices.

The Sponsor will have title to all documentation records, raw data, slides, specimens or other work product generated during the performance of the study. All work product, including raw paper data, pertinent electronic storage media and specimens, will be retained at no charge in the Charles River Archives for a period of 6 months following issuance of the final report. Thereafter, Charles River will charge a monthly archiving fee for retention of all work products, which will be stored in compliance with regulatory requirements. Unless otherwise indicated, any remaining samples (*e.g.*, formulation or blood/plasma/serum) will not be archived, but will be discarded prior to issuance of the final report.

Any work products, including documents, specimens, and samples, that are required by this protocol, its amendments, or other written instructions of the Sponsor, to be shipped by Charles River to another location will be appropriately packaged and labeled as defined by Charles River's SOPs and delivered to a common carrier for shipment. Charles River will not be responsible for shipment following delivery to the common carrier.

Archiving requirements for portions of the study not conducted at Charles River Ashland are described in the applicable appendices.

19 REPORTS

The final report will contain a summary, test and positive control substance information, methods and procedures, appropriate individual animal and summary data tables, Charles River Historical Control Data, supporting sub-reports (analytical chemistry report, *etc.*), a copy of the protocol and amendment(s), if any, and an interpretation and discussion of the study results. The final report will be comprehensive and shall define level(s) inducing toxic effects under the

condition of this investigation. The report will contain all information necessary to conform to current regulatory specifications.

Charles River will submit an electronic copy (PDF and MS Word copy of the report text for editing and comments) of the audited draft report in a timely manner upon completion of data collection prior to issuance of the final report. It is expected that the Sponsor will review the draft report and provide comments to Charles River within a 2-month time frame following submission. Charles River shall provide a revised draft report that incorporates the Sponsor's reasonable revisions and suggestions. One revision will be permitted as part of the cost of the study; additional changes or revisions may be made, at extra cost. Charles River shall submit the final report within 2 weeks of receiving authorization to finalize the report from the Sponsor. If the Sponsor's comments and/or authorization to finalize the report have not been received at Charles River within 6 months following submission of the draft report, Charles River may elect to finalize the report following appropriate written notification to the Sponsor. An electronic copy (bookmarked and hyperlinked PDF) of the final report will be provided; requests for paper copies of the final report may result in additional charges.

20 PROTOCOL MODIFICATION

Modification of the protocol may be accomplished during the course of this investigation. The amendment will identify the section of the protocol to be amended, the amended section, the justification (reason) for the amendment, date of amendment, and signature of the Study Director.

No changes will be made in the study design without the verbal or written permission of the Sponsor. In the event that the Sponsor verbally requests or approves a change in the protocol, such changes will be made by appropriate documentation in the form of a protocol amendment.

21 ANIMAL WELFARE ACT COMPLIANCE

This study will comply with all applicable sections of the Final Rules of the Animal Welfare Act (AWA) regulations (9 CFR) and the current AVMA Guidelines for the Euthanasia of Animals. The protocol will be reviewed by the Charles River Institutional Animal Care and Use Committee (IACUC) prior to commencement of study specific activities. The Sponsor should make particular note of the following:

- The Sponsor Representative's approval of this protocol documents for the Study Director the Sponsor's assurance that the study described in this protocol does not unnecessarily duplicate previous experiments.
- Whenever possible, procedures used in this study have been designed to avoid or minimize discomfort, distress, or pain to animals. All methods are described in this study protocol or in written laboratory Standard Operating Procedures.
- Methods of euthanasia used during this study are in conformance with the above-referenced regulation.

- The Sponsor/Study Director has considered alternatives to procedures that may cause more than momentary or slight pain or distress to the animals and has provided a written narrative description (AWA covered species only) of the methods and sources used to determine that alternatives are not available.

22 REFERENCES

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23 PROTOCOL APPROVAL

Sponsor approval received via e-mail on 06 Oct 2016

Charles River Laboratories Ashland, LLC

Date: 06 Oct 2016.

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